Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis

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Although leukocyte recruitment into alveoli is a hallmark of lung inflammation, the biology of inflammatory cells recruited to the airways is poorly understood in general and only minimally studied in the fetus. Intrauterine infections with inflammation of the chorioamnion (chorioamnionitis) cause fetal lung inflammation and are risk factors for preterm birth (14, 51). Exposure to chorioamnionitis and mechanical ventilation can induce activation of airway inflammatory cells in preterm infants (7, 9, 36). Proteolytic enzymes secreted by activated inflammatory cells have been implicated in the pathogenesis of bronchopulmonary dysplasia (BPD) (1). The recovery of airway leukocytes from preterm infants exposed to chorioamnionitis is only possible for intubated infants after birth. Furthermore, the airway leukocytes are affected by labor and postnatal exposures (e.g., oxygen, ventilation). Consequently, very little is known about airway inflammatory cells in the preterm lung exposed to chorioamnionitis.

We previously (23) used intra-amniotic lipopolysaccharide (LPS) to induce chorioamnionitis and lung inflammation in fetal sheep. Similar to human fetuses exposed to chorioamnionitis, fetal sheep exposed to LPS also develop lung inflammation, have impaired lung alveolar and vascular development, and have increased airway surfactants (2, 20, 23, 51, 52). After intra-amniotic LPS, neutrophils and monocytes are recruited to the fetal air spaces and lung cytokines such as IL-1β and IL-8 increase without evidence of an early oxidative response (8, 23). These inflammatory cells also mediate LPS-induced lung maturation (22). Intra-amniotic LPS also induces functional maturation of fetal lung monocytes to macrophages (30). This maturation of monocytes to macrophages is associated with increased expression of PU.1, a transcription factor for terminal myeloid differentiation (4). These studies were done with monocytes recovered from fetal lung tissue, and there is very little information about the function or fate of inflammatory cells that are recruited to the fetal air spaces. The leukocytes in the air spaces migrate through endothelial and epithelial barriers and therefore may be functionally distinct from lung tissue-associated cells (40). In the fetal mouse lung, LPS increases alveolar type II cells through Toll-like receptor (TLR) 4 and NF-κB (39) and overexpression of NF-κB induces lung maturation and epithelial apoptosis (34). These studies did not evaluate airway leukocytes. The time course of activation, oxidative burst, and apoptosis in airway leukocytes when induced by a proinflammatory stimulus in the fetus is also not known.

NF-κB is a redox-sensitive transcription factor that is critical in the regulation of genes for proinflammatory cytokines and apoptosis (3, 50). In vitro, LPS induces NF-κB activation and primes leukocytes from adult animals and humans to increased oxidative burst activity (12, 43). We hypothesized that intra-amniotic LPS-induced chorioamnionitis would cause activation, maturation, and delayed clearance of fetal leukocytes from the air spaces. The objective of the study was to understand the antecedents of BPD by studying the chorioamnionitis-induced changes in airway leukocytes. We evaluated the activation state, maturation, oxidative-nitrotive stress, antioxidant defenses, and apoptosis (caspase-3 activation) in inflammatory cells recovered by bronchoalveolar lavage (BAL) and in the lung 2 and 7 days after intra-amniotic LPS.
METHODS

Animals and intra-amniotic LPS administration. The sheep experiments were performed in Perth, Western Australia, as approved by the Animal Care and Use Committees of the Cincinnati Children’s Hospital Medical Center and the University of Western Australia. Date-bred Merino ewes were randomized to receive ultrasound-guided intra-amniotic injections with 10 mg of Escherichia coli LPS (055:B5 strain; Sigma, St. Louis, MO) or control injections with equal volumes of saline 15 h before preterm delivery at 124 days of gestational age (term gestation is 150 days). The LPS dose was chosen based on our earlier studies (29), and exposure intervals were based on our previous demonstration (23, 29, 30) that maximal inflammation occurred at 2 days and monocyte to macrophage maturation occurred by 7 days after intra-amniotic LPS.

Delivery and sampling methods. Preterm lambs were surgically delivered, killed with intravascular pentobarbital, dried, and weighed. Umbilical artery blood samples were used for blood gas and pH analyses. The lambs were then exsanguinated; the chest was opened and the trachea cannulated for recovery of a sample of fetal lung fluid and for measurement of a maximal lung volume by air inflation to 40 cm H2O pressure (19). The left lung was lavaged by infusion and aspiration of a sufficient volume (average 50 ml) of normal saline at 4°C to fill the lung (19). The procedure was repeated three times; the BAL fluids (BALFs) were pooled, the total volume was recorded, and aliquots were saved for later analyses. The right upper lobe of the lung was inflation fixed with 10% formalin at 30 cm H2O pressure. Tissue from the right lower lobe was snap frozen in liquid nitrogen, and for measurement of a maximal lung volume by air inflation to 40 cm H2O pressure (19). The left lung was lavaged by infusion and withdrawal of a sufficient volume (average 50 ml) of normal saline at 4°C to fill the lung (19). The procedure was repeated three times; the BAL fluids (BALFs) were pooled, the total volume was recorded, and aliquots were saved for later analyses. The right upper lobe of the lung was inflation fixed with 10% formalin at 30 cm H2O pressure. Tissue from the right lower lobe was snap frozen in liquid nitrogen, and homogenates were prepared with a tissue homogenizer (Omni TH, Marietta, GA) in an appropriate buffer for each assay. Supernatants of lung homogenates were recovered after centrifugation at 5,000 g for 20 min followed by 10,000 g for 10 min at 4°C.

Cells in BALF. BALF samples were centrifuged at 1,500 rpm to recover the cells for quantification and resuspended at the required concentrations for cytospin preparations, nuclear extraction, and flow cytometry analyses. Differential cell counts were performed on cytospins stained with Diff-Quik (Dade Behring, Dudingent, Switzerland). BAL cells from LPS-exposed animals were almost entirely neutrophils and monocytes. Because saline control lambs had very low numbers of inflammatory cells in the BALF, monocytes recovered from dissociated lung tissue of control animals and alveolar macrophages from adult ewes were used in some assays for comparisons (30). Lung monocytes were recovered from dissociated lung tissue on Percoll gradients as described previously (30).

Immunostaining of BALF cytokines and lung tissue. For the cytokine slides, cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 30 min at room temperature. Further fixation and permeabilization was by immersion in ice-cold absolute methanol for 5 min (9). For the lung tissue, a 5-μm paraffin section was used and immunostaining was performed as described previously (20). Endogenous peroxidase was quenched, and antigen epitope retrieval was performed with a heated citrate buffer. The primary antibodies used for cytokine slides were anti-p65, p50 (1:80), PU.1 (1:200), peroxideroxidase V (Prx V) (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) or cleaved caspase-3 (1:100) (Cell Signaling Technology, Danvers, MA). The primary antibodies for lung sections were rabbit polyclonal anti-PU.1 (1:200), goat anti-Prx V (1:100), mouse monoclonal anti-iNOS (1:150) (BD Transduction Labs, San Jose, CA), anti-nitrotyrosine (1:100) (Chemicon International, Temecula, CA), rabbit anti-caspase-3 (1:50), and anti-cleaved caspase-3 (1:100). All primary antibody incubations were performed overnight at 4°C with a blocking serum and were followed by incubation with an appropriate secondary biotinylated antibody. Immunoreactivity was detected with the immunoperoxidase-diaminobenzidine (DAB) method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA), and the cells were counterstained with nuclear fast red. Immunostaining for p65 and p50 NF-κB was evaluated by counting 500 leukocytes in a blinded manner.

Nuclear fraction collection. Nuclear proteins were extracted from BAL cells (2 × 10^6 cells/sample) after minor modifications to the manufacturer’s instructions (Active Motif, Carlsbad, CA). Briefly, the samples were washed in PBS containing phosphatase inhibitors and lysed with hypotonic buffer, followed by further complete lysis with a buffer that included dithiothreitol (DTT) and protease inhibitors. Protein concentration for each nuclear fraction was measured with the Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL).

Electrophoretic mobility shift assay. NF-κB activation of BALF leukocytes was measured by electrophoretic mobility shift assay (EMSA) using the chemiluminescence method (LightShift Chemiluminescent EMSA kit, Pierce). The biotinylated NF-κB oligonucleotide sequences (20 fmol) used were 5’-AGT TGA GGG GAC TTT CCC AGG C-3' and 5’-GCC TGG GAA AGT CCC CTC AAC T-3’. Nuclear extracts (5 μg protein) were electrophoresed on a 6% polyacrylamide minigel with 0.5X Tris-borate-EDTA (TBE) buffer (Novex DNA Retardation Gel, Invitrogen, Carlsbad, CA). Nuclear extracts of preta cells pretreated with 10 nmol TNF-α for 10 min were used as positive controls. Identical biotinylated oligonucleotide sequences in 200-fold excess were the cold probes for competition studies. Supershift studies involved preincubation of samples with 2.5 μg of anti-p65 or anti-p50 antibodies (Santa Cruz Biotechnology) before assay. Detection by chemiluminescence was performed with ECL plus reagents (GE Healthcare, Piscataway, NJ).

Flow cytometry. Leukocytes were first gated based on the expression of CD45 (phycoerythrin-conjugated anti-CD45 antibody, AbD Serotec, Oxford, UK). To determine leukocyte activation, cells from BALF samples were adjusted to a concentration of 1 × 10^6 per 100 μl and labeled with fluorescein isothiocyanate-conjugated anti-CD11b antibody (AbD Serotec). CD11b+ cells above the isotype background intensity were considered as positively stained cells. To assess for oxidative burst activity, BALF samples were adjusted to a concentration of 2 × 10^6 cells per 100 μl. Oxidative burst was measured with the Fc Oxyluc Green assay reagent (Molecular Probes, Eugene, OR), which is based on fluorescence emission generated by oxidation of the Fc receptor-mediated internalization of immune complex. The samples of preta cells pretreated with 10 nmol TNF-α for 10 min were used as positive controls. Identification of leukocytes with oxidative burst activity was performed by cooling samples on ice just before measurement of oxidative burst activity. The measure of baseline oxidative burst activity was a BALF sample kept on ice to block internalization of the reagent complex. Cells showing oxidative burst activity above this baseline were gated. The values were expressed as geometric mean of the fluorescence units normalized to CD45-positive leukocytes.

Assays with fetal lung fluid, BALF, and lung homogenates. Protein concentrations were quantified with the Coomassie (Bradford) protein assay kit (Pierce). Myeloperoxidase (MPO) activity in BALF was determined by measuring the oxidation of tetramethylbenzidine against standard concentrations of pure MPO (Athens Research & Technology, Athens, GA) determined on the basis of its ε₄₅₀ of 91,000 M⁻¹ cm⁻¹ per heme (8). Nitrotyrosine, as a marker of nitrosative stress, was measured in BALF with a competitive enzyme immunosassay (Cell Biolabs, San Diego, CA). The detection sensitivity range was 20 nM to 8 μM of nitrotyrosine-BSA equivalent. Protein carbonyl was measured as a marker of protein oxidation in BALF after derivatization of the samples with dinitrophenylhydrazine (DNPH) and measurement by ELISA (Biocell, Papatoeato, New Zealand) (6, 8). Lipid peroxidation was determined by assaying 8-isoprostane and glutathione peroxidase activity in the fetal lung fluid and lung homogenates [samples were frozen immediately in 0.005% (vol/vol) butylated hydroxytoluene at the time of collection]. The 8-isoprostane assay is an acetylcholinesterase-conjugate tracer-based competitive immunoassay with a lower detection limit of 5 pg/ml (Cayman Chemical, Ann Arbor, MI). Lung homogenates prepared in 5 vols of buffer of 0.1 M

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PBS (pH 7.4), 1 mM EDTA, and 10 μM indomethacin were hydrolyzed with 15% (wt/vol) KOH and affinity-sorbed purified for the measurement of total (free and esterified) 8-isoprostanate according to the manufacturer’s instructions. The use of lung homogenates precludes identification of cellular origin of the oxidant activity. Glutathione peroxidase activity was assessed indirectly when oxidized glutathione (GSSG) produced by the reduction of cumene hydroperoxide by glutathione peroxidase was recycled to its reduced form (GSH) by glutathione reductase and NADPH (Cayman Chemical). NADPH oxidation to NADP⁺ results in a rate of decrease of absorbance at 340 nm directly proportional to glutathione peroxidase activity in conditions when this enzyme is rate limiting (38). Lung homogenates prepared in 5 vols of buffer [in mM: 50 Tris-HCl (pH 7.5), 1 EDTA, and 1 DTT] were diluted to homogenates prepared in 5 vols of buffer [in mM: 50 Tris-HCl (pH 7.5), 1 EDTA, and 1 DTT] were diluted to 0.5 mg/ml protein/sample were subjected to denaturing electrophoresis on a 10–20% Tris-glycine gel (Novex, Invitrogen, Carlsbad, CA) and transferred to nitrocellulose 0.2-μm membranes. The membranes were blocked for nonspecific binding sites with 5% (wt/vol) nonfat milk in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% (vol/vol) Tween 20 (TBS-T) for 1 h at room temperature and incubated overnight at 4°C with a rabbit monoclonal anti-Pu.1 antibody (Cell Signaling Technology) diluted at 1:1,000 in blocking buffer. The membrane was washed with TBS-T and incubated for 1 h at room temperature with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Calbiochem, La Jolla, CA) diluted at 1:20,000 in blocking buffer. Positive controls were extracts from a K562 myelogenous leukemic cell line that have granulocytic and monocytic properties and RAW 264.7 mouse macrophage-like cells. To quantify nitrotyrosine in BALF, aliquots of 40 μg of protein were first dried and reconstituted in smaller volumes of sample buffer before denaturing electrophoresis. For immunoblotting of proteins in lung tissue, 5 vols of ice-cold buffer [in mM: 50 Tris-HCl, pH 7.5, 1 EDTA, 1 DTT, and 1 phenylmethylsulfonyl fluoride (PMSF)] was used for homogenization with the exception of caspase-3 (Chaps Cell Extract Buffer, Cell Signaling Technology). Homogenates were centrifuged at 5,000 g for 20 min and then at 10,000 g for 10 min at 4°C. Samples with 40 μg of protein were loaded for denaturing electrophoresis. The antibodies used for immunoblotting were rabbit polyclonal anti-nitrotyrosine (1:1,000), anti-caspase-3 (1:1,000) (Cell Signaling Technology) and mouse monoclonal anti-Px V (1:1,000) (BD Transduction Labs). Positive controls were from the companies that provided the antibodies except that HeLa cell lysates were used for Px V. Mouse monoclonal antibody against chicken gizzard actin (Seven Hills Bioreagents, Cincinnati, OH) diluted at 1:10,000 and a secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (1:20,000 dilution), were used to reprobe the blots for normalization to β-actin. Detection by chemiluminescence was performed with ECL plus reagent.

Statistics. Statistical analyses were performed with SigmaStat v.1.0 (Jandel, San Rafael, CA). All data are presented as means and SD unless otherwise indicated. For comparisons of more than two groups, ANOVA followed by Student-Newman-Keuls tests for post hoc analyses were used. For analyses of nonparametric data, Kruskal-Wallis one-way ANOVA on ranks and Dunn’s test as a multiple-comparison method were used. Selective comparisons of two groups were made with unpaired t-tests or Mann-Whitney U-tests as indicated. Significance was accepted at P < 0.05.

RESULTS

Description of lambs. There were no fetal deaths as a result of the intra-amniotic injections or LPS exposures, and the animals did not have metabolic acidosis at delivery. The maximal lung volumes measured at 40 cmH₂O air pressure were low for control animals and at 2 days but increased at 7 days, indicating induced lung maturation (Table 1).

Markers of inflammation in BALF. More neutrophils than monocytes appeared in the BALF in response to intra-amniotic LPS, and there were more inflammatory cells at 2 days than at 7 day. BALF of saline control animals was devoid of inflammatory cells. CD11b expression is a marker of mature activated neutrophils and monocytes (42). Flow cytometry analysis of CD45 inflammatory cells in BALF from LPS-exposed animals demonstrated that 4–6% of these were CD11b positive. The mean percentages of CD11b-positive leukocytes in these lambs were similar to those in alveolar macrophages from adult ewes (data not shown) but significantly higher than lung monocytes (1%) from non-LPS-exposed fetal lambs (Table 1). Consistent with the large neutrophil influx in the BALF 2 days after exposure, MPO activity, a measure of neutrophil activation, increased 20-fold, and the total protein increased 2-fold (Table 1).

NF-κB activation in BALF leukocytes. Of the animals exposed to LPS, six in the 2-day group and five in the 7-day group had samples with sufficient BAL leukocytes for nuclear extract analyses for NF-κB activation with EMSA. Saline control animals demonstrated no activation of NF-κB (lane 2, Fig. 1A). All the samples in the 2-day LPS-exposed group had intense NF-κB activation (lanes 3 and 4, Fig. 1A). In contrast, in the 7-day LPS group, three of five samples showed activation (lane 5) and two were negative (40%; lane 6) (Fig. 1A). BALF samples from three adult ewes not exposed to LPS had less activated NF-κB complexes compared with samples from LPS-exposed lambs (compare lane 7 with lanes 3–5, Fig. 1A). Supershift assays using either anti-p65 or anti-p50 antibodies demonstrated almost complete attenuation of the NF-κB bands (lanes 8 and 9, Fig. 1A), indicating that both of these subunits were present in the nuclei of the activated leukocytes. Additionally, p65 and p50 subunits of NF-κB were present in the nuclei of both neutrophils and monocytes by immunocytochemistry (Fig. 1, B, C, and E). More neutrophils than monocytes had either p65 or p50 NF-κB activation 2 days after LPS exposure (P = 0.03) (Fig. 1F). At 7 days after LPS exposure, p65 NF-κB staining was localized to the cytoplasm in many leukocytes, indicating an inactive com-

Table 1. Description of animals and markers of inflammation in alveolar lavage fluid

<table>
<thead>
<tr>
<th></th>
<th>Saline Control (n = 9)</th>
<th>2-day LPS (n = 9)</th>
<th>7-day LPS (n = 7)</th>
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</thead>
<tbody>
<tr>
<td>Animals</td>
<td></td>
<td></td>
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<tr>
<td>Birth weight, kg</td>
<td>2.9 (0.3)</td>
<td>2.6 (0.2)</td>
<td>2.3 (0.2)*</td>
</tr>
<tr>
<td>Lung volumes at 40 cmH₂O, ml/kg</td>
<td>7.0 (2.5)</td>
<td>5.9 (4.9)</td>
<td>26.4 (6.6)*</td>
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<tr>
<td>BALF measurements</td>
<td></td>
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<tr>
<td>Neutrophils, 10⁶/ml</td>
<td>0.05 (0.13)</td>
<td>356 (213)*</td>
<td>116 (237)</td>
</tr>
<tr>
<td>Monocytes, 10⁶/ml</td>
<td>0.11 (0.23)</td>
<td>112 (75)*</td>
<td>36 (53)</td>
</tr>
<tr>
<td>CD11b-positive leukocytes, %</td>
<td>1.2 (1.1)</td>
<td>6.5 (2.3)*</td>
<td>4.2 (1.6)*</td>
</tr>
<tr>
<td>Protein, mg/ml</td>
<td>0.17 (0.06)</td>
<td>0.38 (0.13)*</td>
<td>0.24 (0.14)</td>
</tr>
<tr>
<td>Myeloperoxidase, pM</td>
<td>4 (10)</td>
<td>83 (28)*</td>
<td>49 (67)</td>
</tr>
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</table>

Values are means (SD) for n animals; CD11b-positive leukocytes from lung monocytes in saline control animals (n = 5) and 2-day lipopolysaccharide (LPS)-exposed animals (n = 6). BALF, bronchoalveolar lavage fluid. *Statistically significantly by ANOVA vs. control animals.
plex (Fig. 1D). Both neutrophils and monocytes had more activated p50 than p65 NF-κB 7 days after LPS exposure

(P < 0.01) (Fig. 1F).

**PU.1 expression in BAL leukocytes.** Neutrophils and monocytes in BALF from 2-day LPS-exposed animals had nuclear PU.1 immunostaining (Fig. 2A). Similarly, neutrophils and monocytes from the 7-day LPS group also stained positively for PU.1, and there were many macrophages with intense nuclear PU.1 staining (Fig. 2B). Relative to the 2-day group, nuclear extracts of the 7-day LPS group had increased PU.1 expression by Western blot (Fig. 2, C and D). The expression was similar to PU.1 in alveolar macrophages from adult ewes.
Markers of oxidative-nitrative stress in BALF. Compared with lung cells from control animals, leukocytes in BALF from the 2-d and the 7-d LPS-exposed groups had a higher oxidative burst (Fig. 3A). Consistent with our previous study (8), protein oxidation assessed by carboxyls in the BALF increased eightfold 7 days after LPS exposure (Fig. 3B). However, there was minimal lipid peroxidation in the BALF in response to intra-amniotic LPS. Free 8-isoprostanate levels increased fivefold in the 2-day-exposed group (Fig. 3C). However, total 8-isoprostanate in lung homogenates was low and not different between groups (mean 47 ± 19 pg/ml). Similarly, glutathione peroxidase activity as a marker of free fatty acid oxidation was below the assay detection range. Nitrotyrosine as a marker of protein nitration was not detected in BALF by either ELISA or Western blotting (data not shown). To further search for nitration activity, lung sections were stained for inducible nitric oxide synthase (iNOS) (Fig. 4). Inflammatory cells in LPS-exposed animals were positive for NOSII (iNOS) (Fig. 4B). The lack of protein nitration in the lung could not be attributed to endogenous denitrase activity, since incubation of supernatants from lung homogenates with nitrotyrosine-modified albumin did not decrease nitrotyrosine levels. These results demonstrate minimal oxidative stress after intra-amniotic LPS.

Leukocyte and lung antioxidant enzymes. Airway neutrophils and monocytes from LPS-exposed animals (2-day and 7-day groups) stained positively for Prx V. Prx V was detected mainly in the perinuclear cytoplasm, with occasional intranuclear staining (Fig. 5A). Immunolocalization of Prx V in the lung showed a low abundance in the mesenchyme of the peribronchial epithelial region (Fig. 5B), compared with a greater overall staining contributed by inflammatory cells. There was a trend toward higher expression of Prx V in the lung homogenates of the 7-day LPS-exposed group (P = 0.07) (Fig. 5, C and D). Lung homogenate glutathione peroxidase enzyme activity was also elevated in the 2-day and 7-day LPS-exposed animals compared with saline control animals (409 ± 43 and 409 ± 62 vs. 305 ± 54 nmol·min⁻¹·ml⁻¹, respectively; P < 0.001).

Leukocyte and lung epithelial apoptosis. The BAL cells had a low rate of apoptosis assessed by cleaved caspase-3 immunostaining (Fig. 6). The percentage of cells with cleaved caspase-3 were 4% and 6% in the 2-day and 7-day LPS groups, respectively. Cleaved caspase-3 was not detected in the lung epithelium, and only an occasional inflammatory cell in the air spaces was positive (Fig. 6B). In contrast, there was abundant staining of the inactive form of caspase-3 (Fig. 6, C and D). The majority of the multilobed nuclei of neutrophils also lacked features of nuclear shrinkage suggestive of apoptosis (Fig. 6E). This finding was further substantiated by Western blot analysis of lung homogenates showing similar amounts of inactive caspase-3 between groups but absence of the cleaved caspase-3 in all the groups (Fig. 6, F and G).

DISCUSSION

Chorioamnionitis resulting in fetal inflammation is a risk factor for BPD (48). Postmortem analyses of fetal deaths associated with chorioamnionitis showed lung inflammation characterized by heavy leukocyte infiltrates and upregulated cytokines (44). Other risk factors for BPD such as mechanical ventilation and hyperoxia also can cause inflammation (15, 18). Increased inflammatory cells, delayed clearance of inflamma-
and oxidative burst activity but had minimal apoptosis. After recruitment to the air spaces, the fetal leukocytes expressed the maturation and differentiation factor PU.1.

Chorioamnionitis is caused by multiple different organisms isolated from the amniotic fluid (37). Unlike a robust lung inflammation induced by intra-amniotic injection of E. coli LPS (TLR4 agonist) in the fetal sheep, TLR2 or TLR3 agonists caused minimal lung inflammation (16). Despite the heterogeneity of organisms in human chorioamnionitis, infants exposed to chorioamnionitis and fetal sheep exposed to intra-amniotic LPS had similar pathophysiological consequences. Both of these groups have impaired lung alveolar and vascular development and have increased airway surfactants (2, 20, 23, 51, 52). The sequence of activation and apoptosis of fetal lung cells after exposure to inflammatory stimuli other than TLR4-activating agents needs to be evaluated.

Neutrophils and monocytes recruited to the fetal lung after exposure to LPS were associated with upregulation of various proinflammatory cytokines in the lung (29). However, we did not know whether these cells were activated and could contribute to the amplification of the inflammation. Studies in preterm infants exposed to chorioamnionitis linked airway leukocyte NF-κB activation with increased cytokines (9). NF-κB is a crucial transcription factor that regulates many proinflammatory cytokine genes (3). The results of this study showed that both neutrophils and monocytes had NF-κB activation at 2 days and persistent activation at 7 days after exposure to LPS. Of note, the p65 subunit of NF-κB was primarily expressed in neutrophils at 2 days compared with 7 days, while both neutrophils and monocytes predominantly expressed p50 and not p65 NF-κB at 7 days. The precise roles of the different subunits of NF-κB are unclear, although the classical NF-κB activation pathway (p65/p50 heterodimer) is known to be primarily involved in the transcriptional regulation of proinflammatory genes. Recently, Lawrence et al. (32) reported that the p50 homodimer may be associated with the repression of inflammation. The role of the dynamic changes in NF-κB subunit activation in air space leukocytes in the modulation of inflammation and resolution in the fetus remains to be studied.

PU.1 is a transcription factor that is a critical regulator of myeloid cell differentiation, particularly during the terminal differentiation of macrophages (26, 45, 49). PU.1 was not detected in mature murine neutrophils but was expressed at high levels in mature human blood monocytes and neutrophils (10). The alveolar macrophages of newborn rats terminally differentiate ~15 days after birth, with concomitant increase in PU.1 expression (17). We recently reported (30) that fetal lung monocytes stimulated with LPS-induced chorioamnionitis matured to macrophages with increased expression of PU.1. In the present study, we provided further evidence that both BAL neutrophils and monocytes from fetal sheep exposed to LPS expressed PU.1 within 2 days of LPS exposure. Ours is the first documentation that airway neutrophils express PU.1, indicating that neutrophils recruited during fetal lung inflammation are terminally differentiated.

Some leukocytes recovered from the airways were activated as more neutrophils and monocytes from LPS-exposed lambs expressed CD11b than from saline control lambs, with a level of expression similar to that of mature alveolar macrophages from the adult ewe. One measure of functional maturation of

![Graph A](image1.png)

**Fig. 3. Oxidant activity in BALF after intra-amniotic LPS.** A: BAL leukocyte oxidative burst was measured by flow cytometry and expressed as geometric mean of fluorescence in the CD45+ cells normalized to cell numbers. B: BALF protein carbonyl. C: 8-isoprostane levels in the fetal lung fluid. Control samples in A were lung monocytes isolated on Percoll gradients. Lipid peroxidation increased at 2 days, oxidative burst activity increased at 2 days and 7 days, and protein oxidation increased 7 days after LPS. *P < 0.05 vs. control. n = 5 or 6 animals/group.
leukocytes is their ability to activate oxidant pathways. Consistent with observations in LPS-exposed neutrophils (53), the oxidative burst activity was higher in fetal leukocytes in BALF of LPS-exposed animals compared with control animals. Consistent also with our previous findings, protein carbonyl as a marker of oxidative stress was only detectable in BALF at 7 days after LPS exposure (8). The time profile for the increase in protein carbonyl in this study suggests that maturation of leukocytes contributes to increased activity. Compared with protein oxidation, there was undetectable protein nitration in the BALF. Although systemic endotoxin exposure in adult rats increased a nitrotyrosine-modifying lung denitrase factor (24), we found negligible denitrase in our model. Despite the absence of this endogenous denitrase activity, nitrotyrosine was not detected in the BALF and lung homogenates. However, the airway inflammatory cells stained strongly for NOSII (iNOS). There was a small increase in lipid peroxidation induced by exposure to intra-amniotic LPS. For comparison, the highest level of 8-isoprostane in this study (35 pg/ml) was at the low end of levels reported in adult patients with inflammatory lung disorders (25). Despite the expression of activation markers and oxidants in the fetal leukocytes, the overall oxidative stress to the fetal lung in response to intra-amniotic LPS was minimal.

Peroxiredoxins are a group of potent antioxidant defense enzymes involved in scavenging reactive oxygen species and hydrogen peroxide-mediated cell signaling (54). Of the six peroxiredoxins, Prx V is a major antioxidant defense in migratory leukocytes during airway inflammation (31). Prx V also possesses peroxynitrite reductase activity, and it may protect leukocyte autooxidation from NOS-mediated peroxynitrite generation (13). Prx V is also an inhibitor of p53-dependent apoptosis (55). We found that airway leukocytes contributed to Prx V expression in the inflamed fetal lung. Prx V was mainly concentrated in the cytoplasmic region and was present in both BAL neutrophils and monocytes. The lung parenchyma showed mild staining in the peribronchial mesenchyme by immunolocalization of Prx V. Furthermore, consistent with our previous findings (46), intra-amniotic LPS also increased another lung antioxidant enzyme, glutathione peroxidase. These results suggest that the preterm fetus mounted a modest antioxidant response to the LPS challenge.

![Fig. 4. Inducible nitric oxide synthase (iNOS, NOSII) expression after intra-amniotic LPS exposure: immunostaining on paraffin-embedded lung sections using an anti-iNOS antibody in control (A) and 2-day LPS (B) groups. Positive immunoreactivity is shown by dark brown staining of the inflammatory cells (arrow). n = 4/group. Bar, 50 μm.](image)

![Fig. 5. Peroxiredoxin V (Prx V) expression after intra-amniotic LPS. A: Prx V immunostaining using BALF cytospin leukocytes from a 7-day LPS-exposed animal shows immunoreactivity in neutrophils and monocytes with dark brown staining localized mainly in the cytoplasm (arrow) and rarely with nuclear staining (arrowhead). B: lung section from a saline control animal shows Prx V localization in the peribronchial mesenchyme (arrows). Bar, 50 μm. C and D: representative Western immunoblot for Prx V and β-actin (C) and quantification (D) for Prx V in lung homogenates; n = 6 or 7/group. Lung homogenate Prx V did not significantly increase after LPS exposure. +, Positive control using HeLa cell lysates.](image)
protective mechanism against the small inflammation-induced oxidative stress.

Necrosis of leukocytes causes tissue injury secondary to the release of toxic metabolites and enzymes. In contrast, programmed cell death or apoptosis leads to the clearance of inflammatory cells by phagocytes without such consequences. Delayed or diminished leukocyte apoptosis is known to occur during inflammation (33). We previously reported (28) that fetal lambs exposed to intra-amniotic LPS had a low apoptosis rate (2–6% of cells) in the lung parenchyma or inflammatory cells as measured by terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL). The present study further confirmed that there was minimal apoptosis in the airway leukocytes (4–6% of the cells) and the lung parenchyma within 7 days of exposure to intra-amniotic LPS, as measured by cleaved caspase-3 expression. Our findings are contrary to the results by May et al. (35) in their postmortem analysis of fetal deaths, which showed extensive apoptosis in alveolar epithelium of fetuses exposed to chorioamnionitis. However, their results were based on fetal lungs at different developmental stages, likely exposed to multiple inflammatory insults for prolonged periods. Consistent with the minimal leukocyte apoptosis in this study, the BAL cells had persistent activation of NF-κB, a known regulator of antiapoptotic genes (50).

Intracellular oxidants are known to induce apoptosis (41). A greater increase in antioxidant activity relative to a more modest oxidative stress within the airway leukocytes may have contributed to the minimal airway inflammatory cell apoptosis in this study. The lack of significant oxidants within the BALF correlated with minimal apoptosis in the lung parenchyma. Regardless of the mechanism, these results demonstrate that, unlike the adult (5, 11), inflammatory cells recovered from the air space of the fetus have a delayed clearance.

Our results provide insights into the effects of chorioamnionitis on the fetal lung. We previously reported (21, 27) that prenatal exposure to chorioamnionitis induced by LPS can...
either augment or inhibit (tolerance response) the responsiveness of lung and blood monocytes to a variety of TLR agonists. The oxidative-nitrative stress in the relatively hypoxic intrauterine environment was modest and unlikely to contribute substantially to lung injury. However, the chorioamnionitis-induced persistent activation, maturation, and delayed clearance of fetal leukocytes from the air spaces may contribute to exaggerated injury responses to postnatal inflammatory insults such as mechanical ventilation and nosocomial sepsis.

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